# STAT5 interferes with PD-1 transcriptional activation and affects CD8+ T-cell sensitivity to PD-1-dependent immunoregulation

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## Abstract

**Programmed cell death-1 (PD-1) is a co-inhibitory receptor that dampens immune responses upon interaction with PD-L1 and PD-L2. Although PD-1 expression on T cells is known to be activation-dependent, how cytokines modify its regulation is not fully resolved. Using polyclonal T-cell activation to study cytokine-dependent PD-1 regulation, we found that IL-2 inhibited transcriptional up-regulation of PD-1 despite the promotion of T-cell activation. The IL-2-mediated reduction in PD-1 expression augmented CD8+ T-cell activities against PD-L1-expressing target cells. To study the mechanism of PD-1 reduction, we focused on STAT5 activation in the IL-2 signaling pathway. Bioinformatic analysis suggested a novel conserved PD-1 promoter domain where NFAT and STAT5 can potentially compete with each other for binding. NFAT1 interaction with this domain revealed substantial potency in PD-1 transcription compared to STAT5A, and STAT5A overexpression could quench NFAT1-dependent PD-1 up-regulation in a sequence-specific manner. Chromatin immunoprecipitation analysis of activated T cells showed that IL-2 treatment significantly diminished the binding of NFAT1 and NFAT2 in the hypothesized competition site, while STAT5 binding to the same region was increased. These results raise the possibility that the competition of transcriptional factors might be involved in the fine-tuning of PD-1 expression by cytokines such as IL-2.**

**Graphical Abstract**
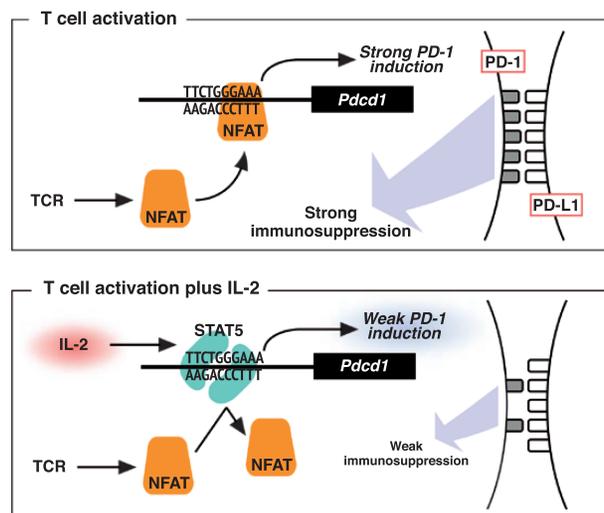


*Keywords:* IL-2, immune checkpoint, immunosuppression, NFAT, tumor microenvironment

## Introduction

Programmed cell death-1 (PD-1), which carries two tyrosine-based inhibitory motifs (ITIM and ITSM) with its cytoplasmic tail, is a transmembrane protein that shares homology with the B7/CD28 family of T-cell surface molecules ([1]). PD-1 interacts with its physiological ligands PD-L1 and/or PD-L2, providing negative regulatory signaling for T-cell proliferation, cytokine production and antigen clearance. PD-1 down-regulates immune activities by recruiting the non-receptor type tyrosine phosphatase Src homology 2 domain-containing tyrosine phosphatase-2 (SHP-2), which dephosphorylates T-cell receptor (TCR) proximal signaling intermediates ([2], [3]).

Genetic deletion of the PD-1 gene in mice spontaneously leads to the development of various autoimmune diseases such as arthritis, gastritis and cardiomyopathy ([4], [5]). In addition, when PD-1 was inactivated, the induction of inflammatory diseases resulted in exacerbated outcome as shown by the accelerated onset and enhanced severity ([6], [7]). In contrast, T cells expressing PD-1 at high levels are reluctant to get rid of pathogens during chronic viral infection or tumor progression ([8], [9]). Together, these manifestations confirm the suppressive activity of PD-1 as an essential negative regulator for the development and maintenance of peripheral tolerance, and also motivate the administration of blocking antibodies against PD-1 or its ligands in rejuvenation of exhausted T-cell functions.

Along with the success of anti-PD-1 antibodies (e.g. nivolumab, pembrolizumab) in clinical applications against cancer, the mechanisms of PD-1 regulation have been extensively studied. Following the initial research on the induction of PD-1 transcription by NFAT2 from TCR signaling ([10], [11]), several other factors involved in PD-1 up-regulation were identified in functionally anergic T cells or T-cell lines with their transcription factor binding sites mapped onto the PD-1 promoter locus. FOXO1 in persistent infection, TGF-β in the tumor microenvironment and IRF9 under IFN-α signaling are involved in PD-1 up-regulation ([12–15]). However, the transcriptional down-regulation of PD-1 during primary T-cell activation was not fully understood.

In this study, we examined the changes in PD-1 expression levels by various cytokines in the polyclonal activation of primary T cells. We found that PD-1, but not LAG3, was transcriptionally down-regulated by IL-2 signaling. Bioinformatic analysis identified an overlapped binding site of NFAT and STAT5 within the conserved region of the PD-1 promoter. Indeed, activated STAT5A could compete with NFAT1 at this site and quench NFAT1-dependent PD-1 up-regulation. Activated STAT5 in IL-2-treated T cells occupied the hypothesized competition site and prevented the binding of NFAT1 and NFAT2. These results suggest STAT5 as a direct modulator of PD-1 expression during T-cell activation.

## Methods

### Mice

C57BL/6JJmsSlc mice were purchased from Japan SLC (Hamamatsu, Japan). C57BL/6 PD-1-KO mice were obtained from RIKEN BioResource Research Center (Tsukuba, Japan). All mice were maintained under specific pathogen-free conditions at the Kobe BM Laboratory (Oriental Bio Service, Kobe, Japan).

### Cells

Primary mouse T cells, DO11.10 T-cell hybridoma and the IIA1.6 FcR$^+$ PD-L1$^+$ cell line were maintained in RPMI 1640 (Wako, Osaka, Japan), and HEK293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose) (Nacalai Tesque, Kyoto, Japan). Media (500 ml) was supplemented with 10% fetal bovine serum (FBS; Biosera, Kansas City, MO, USA), 0.89 g HEPES (Dojindo, Tokyo, Japan), 7.5 ml penicillin–streptomycin solution (Cat. 16823191, Wako), 7.5 ml L-glutamine solution (Cat. 073-05391, Wako), 3.75 ml sodium pyruvate solution (Cat. 190-14881, Wako), 3.75 ml non-essential amino acids solution (Cat. 139-15651, Wako), 3.75 mg gentamicin sulfate (Wako) and 2.6 μl 2-mercaptoethanol (Wako).

### Cytokines

Recombinant murine IL-2, IL-6, IL-12, IFN-γ and human TGF-β1 were obtained from PeproTech (Cranbury, NJ, USA). Recombinant murine IL-4 was obtained from Miltenyi Biotec (Auburn, CA, USA), IL-18 was from BioLegend (San Diego, CA, USA) and IL-23 was from R&D Systems (Minneapolis, MN, USA). All cytokines were used at 10 ng ml$^{-1}$ except for specific indications.

### Antibodies

The following monoclonal antibodies (mAbs) were purchased from BioLegend: PE-anti-CD25 (PC61), APC-anti-CD69 (H1.2F3), BV421-anti-PD-1 (29F.1A12), fluorescein isothiocyanate (FITC)- and PerCP/Cy5.5-anti-CD8a (53-6.7), Ultra-LEAF purified anti-mouse CD3ε (145-2C11), Ultra-LEAF purified anti-mouse CD28 (37.51) and purified anti-mouse PD-1 (29F.1A12) mAbs. Anti-NFAT1 (D43B1), NFAT2 (D15F1) and STAT5 (D2O6Y) mAbs were from Cell Signaling Technology (Danvers, MA, USA). eBioscience Fixable Viability Dye was from ThermoFisher (Waltham, MA, USA).

### T-cell culture

CD8$^+$ or CD4$^+$ T lymphocytes (>95% purity) were purified by negative sorting using AutoMACS (Miltenyi Biotec) and were stimulated with a T Cell Activation/Expansion Kit (Miltenyi Biotec) in the presence or absence of cytokine for 28 h. For the reculturing, IL-2-primed CD8$^+$ T cells were washed using RPMI for three times before re-stimulation with anti-CD3ε and CD28 mAbs using MACSiBeads (Miltenyi Biotec) in a 1:1 ratio for 12 h.

### Flow cytometry analysis

For surface staining, cells were washed with 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) twice and stained at 4°C for 20 min. Data were acquired by a LSRFortessa X-20 (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo v10.1 (Treestar, Ashland, OR, USA).

*PD-1-mediated T-cell inhibition (cytotoxicity)*

Activated CD8⁺ T cells (effector cells, E) were washed with media three times and then co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled FcR⁺ PD-L1⁺ IIA1.6 cells (target cells, T) at various E:T ratio in a 96-well v-bottom plate with 2 µg ml⁻¹ anti-CD3ε mAb, 2 µg ml⁻¹ anti-CD28 mAb and 1 µg ml⁻¹ anti-PD-1 mAb for 3 h. DO11.10 T-cell hybridoma was added before the flow cytometric data acquisition as a reference after staining with PE-anti-DO11.10 TCR mAb. The percentage killing was calculated as the ratio of target cells to reference cells after normalization with the E:T = 0:1 result.

*PD-1-mediated T-cell inhibition (cytokine)*

CD8⁺ T cells were stimulated for 28 h in the presence of IL-2 and received re-stimulation with the same beads in the presence or absence of IL-2 for 12 h. Subsequently, activated CD8⁺ T cells were washed with media three times and IFN-γ-producing activities of T cells were evaluated in a co-culture with FcR⁺ PD-L1⁺ IIA1.6 cells in a 1:1 ratio in a 96-well plate with 2 µg ml⁻¹ anti-CD3ε mAb, 2 µg ml⁻¹ anti-CD28 mAb and 1 µg ml⁻¹ anti-PD-1 mAb for 6 h. IFN-γ levels in the supernatant were determined using a mouse IFN-γ DuoSet ELISA Kit (R&D Systems). T cells co-cultured with PD-L1-expressing IIA1.6 cells were subjected to intracellular cytokine staining. The cells were stimulated for 4 h followed by further incubation for 2 h after adding brefeldin A (10 µg ml⁻¹). Cells were fixed in 4% paraformaldehyde in PBS at 4°C for 15 min, washed with 1% BSA in PBS, and permeabilized with buffer containing 10 mM Tris, 5 mM EDTA, 50 mM NaCl and 0.5% Triton X-100 at 4°C for 15 min. After washing, cells were incubated with FITC-labeled anti-IFN-γ mAb (XMG1.2) at 4°C for 30 min.

*Quantitative PCR*

Total RNAs from activated CD8⁺ T cells were extracted by an RNeasy mini kit (Qiagen, Hilden, Germany). One microgram of the total RNA was then subjected to reverse transcription using the PrimeScrip II 1st Strand cDNA synthesis kit (Takara, Kusatsu, Japan). The PD-1 and FBXO38 transcripts were quantified by the CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using the SsoAdvance Universal SYBR Green Supermix (Bio-Rad). PD-1 and FBXO38 expression was normalized to the 18S ribosomal RNA. Primers used were: Mouse Pdcd1, forward 5′-CTACCTCTGTGGGGCCATC-3′, reverse 5′-GAGGTCTCCAGGATTCTCTCTGT-3′; Mouse Fbxo38, forward 5′-ATGGGACCACGAAAGAAAAGTG-3′, reverse 5′-TAGCTTCCGAGAGAGGCATTC-3′; 18s ribosomal RNA, forward 5′-GTAACCCGTTGAACCCCATT-3′, reverse 5′-CCATCCAATCGGTAGTAGCG-3′.

*Transcription factor prediction and conserved region search*

The prediction of transcription factors was performed using the online database JASPAR2020 (http://jaspar.genereg.net). The conserved region was searched by Multiz Alignments of 60 Vertebrates on the UCSC Genome Browser.

*Mouse PD-1 reporter constructions*

The DNA fragment containing the upstream region and 5′ untranslated region (UTR) of the B6 mouse PD-1 gene was cloned into the SacI-XhoI site of the pGL4.12 vector (Promega, Madison, WI, USA) for promoter analyses. The pRL-SV40 vector (Promega) was used as an internal control for monitoring the transfection efficiency.

*Constitutively active NFAT1 and STAT5A constructions*

The constitutively active NFAT1 (NFAT1-CA) and constitutively active STAT5A (STAT5A-CA) were designed by cloning the constitutively active NFAT1 sequence (plasmid #11792, Addgene, Watertown, MA, USA) and constitutively active STAT5A sequence (plasmid #83255, Addgene) into the mammalian expression system pSELECT (InvivoGen, San Diego, CA, USA) to produce pSELECT-NFAT1-CA and pSELECT-STAT5A-CA vectors.

*Luciferase assay*

The transfection process was conducted using FuGENE HD (Promega). Generally, HEK293FT cells were plated at a density of 2 × 10⁴ cells per well of a 96-well plate in 100 µl of complete growth medium (DMEM + 10% FBS) 24 h before the transfection. The pGL4.12 PD-1 reporter vector (5 ng) and pRL-SV40 control vector (5 ng) together with various amounts of pSELECT-NFAT1-CA and pSELECT-STAT5A-CA were mixed with 0.3 µl of FuGENE HD reagent and OptiMEM (ThermoFisher Gibco). The mixture (5 µl) was introduced to each well after 5- to 10-min incubation at room temperature. After 24 h, the luciferase activity was measured by the Dual-Glo Luciferase Assay System (Promega) using a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). The PD-1 promoter activity was normalized to the Renilla luciferase activity.

*Site-directed mutagenesis*

The native CR −100 bp Luc Reporter vector was mutated by a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) to generate a mutant CR −100 bp Luc Reporter vector using the primers: forward 5′-CCTGCTTTGAAACATTTCCCATTTGCTAAAGGTACCGGCCAGTTAG-3′, reverse 5′-CTAACTGGCCGGTACCTTTAGCAAATGGGAAATGTTTCAAAGCAGG-3′ designed by QuikChange Primer Design (https://www.chem.agilent.com/store/primerDesignProgram.jsp?toggle=uploadNow&mutate=true&_requestid=397581).

*Chromatin immunoprecipitation–quantitative PCR*

The experiment was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). T cells stimulated with or without IL-2 were cross-linked with 1% formaldehyde at room temperature for 10 min. Fragmented chromatin was treated with nuclease and subjected to sonication. Chromatin immunoprecipitation (ChIP) was performed with anti-NFAT1, anti-NFAT2, anti-STAT5 mAb or the same amount of normal rabbit IgG. After reverse cross-linking and DNA purification, immunoprecipitated DNA was quantified by
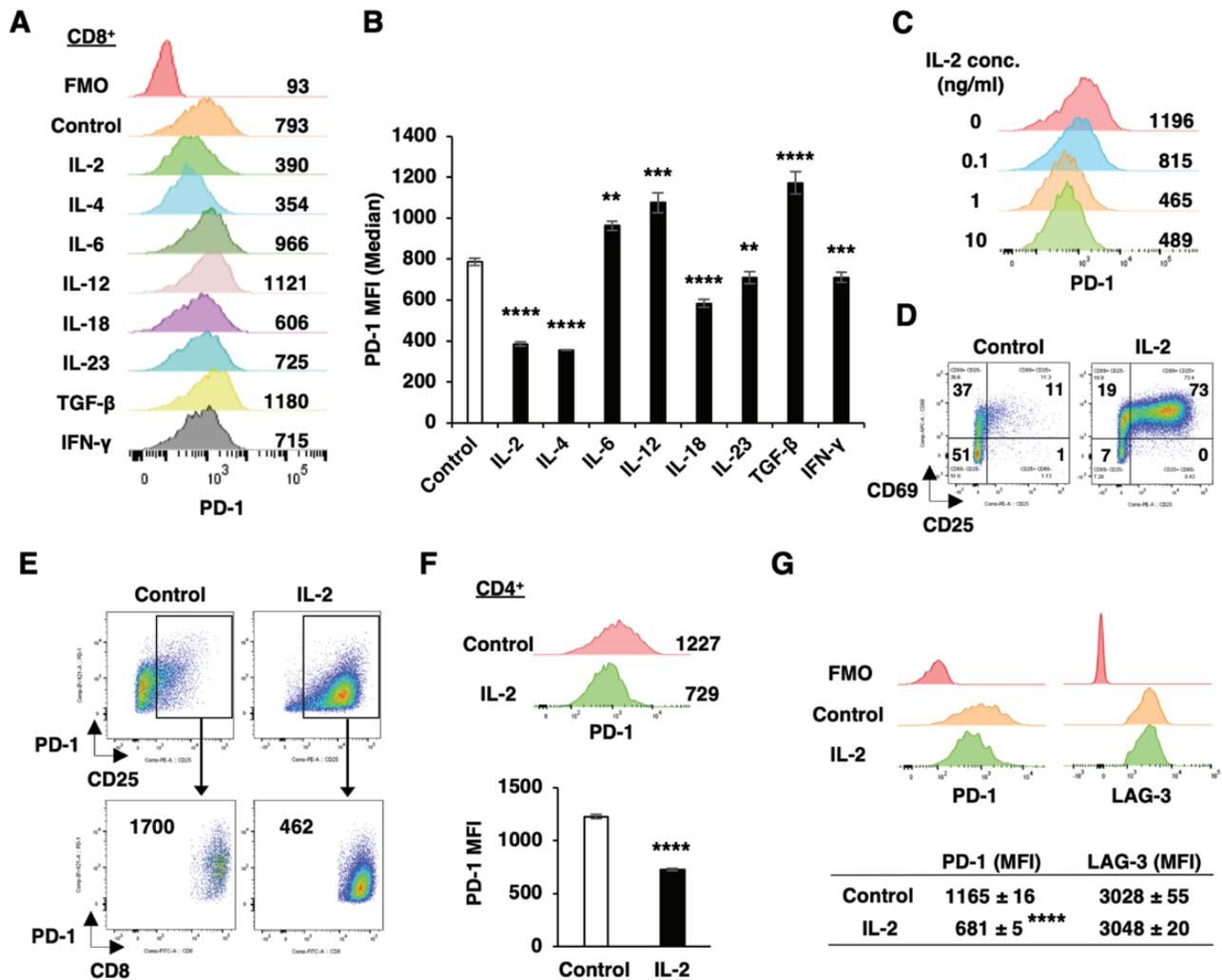
**Fig. 1.** Cytokines modify PD-1 expression in activated T cells. (A, B) PD-1 expression on mouse CD8+ T cells after stimulation with anti-CD3 and anti-CD28 mAbs for 28 h in the presence of various cytokines (10 ng ml$^{-1}$). Numbers in the panels represent mean fluorescence intensity of PD-1 signal. (C) Dose-dependent reduction of PD-1 by increasing concentrations of IL-2. (D) IL-2 promoted T-cell activation as indicated by up-regulation of CD25 and CD69. (E) PD-1 reduction by IL-2 was also observed in the CD8+ CD25+ population. (F) IL-2 reduced PD-1 induction in stimulated CD4+ T cells. Sorted mouse CD4+ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 28 h in the presence or absence of IL-2. (G) IL-2 interfered with PD-1 up-regulation in activated CD8+ T cells, but it did not affect LAG3 levels. Data represent the means ± SEM. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, two-tailed student's $t$-test analysis. Data shown here are representative of three independent experiments.

real-time PCR using the SsoAdvance Universal SYBR Green Supermix. Primers for PD-1 conserved region B (CR-B) were forward 5′- GGCAGTGTCGCCTTCAGTAGC-3′, reverse 5′-CCACCTCTAGTTGCCTGTTCTC-3′; PD-1 conserved region C (CR-C) is forward 5′- CCTCACCTCCTGCTTGTCTCTC-3′, reverse 5′- GTGAGACCCACACATCTCATTGC-3′; and PD-1 negative control (N/A) is forward 5′- TCATGTGTATTGGGGGATT GCGTGTG-3′, reverse 5′- CAGCACACATGATAGAGCCC ACACA-3′. The percent input was calculated by the following formula: percent input = 2% × 2(C[T] 2% input sample − C[T] IP sample); C[T] = threshold cycle of PCR reaction, IP: immunoprecipitated.

*Statistical analysis*

All experiments were performed at least three times. Results are expressed as mean ± SEM. and analyzed by two-tailed student's $t$-test or one-way analysis of variance. The $P$ value <0.05 was considered statistically significant. The analysis was conducted using the Graphpad 8.4.3 software.

## Results

*IL-2 reduces the PD-1 expression level in activated T cells*

In order to understand the effects of cytokines on PD-1-dependent immunoregulation, we stimulated purified mouse CD8+ T cells with anti-CD3 and anti-CD28 mAbs in the presence of various cytokines. After 28 h, stimulated T cells up-regulated PD-1 expression on their surface. Interestingly, treatment with IL-2 or IL-4 significantly reduced PD-1 levels, while IL-12 and TGF-β further enhanced PD-1 expression (Fig. 1A and B). The same trend of cytokine-dependent PD-1 regulation persisted on day 2 (Supplementary Figure 1). PD-1

up-regulation is considered a negative feedback mechanism to prevent overactivation of T cells. However, while IL-2 clearly promotes T-cell activation, the reduction of PD-1 expression by this cytokine seems to prevent the negative feedback in immune activation. This IL-2-dependent negative regulation of PD-1 may be biologically active since *in vivo* IL-2 treatment of mice was previously reported to reduce PD-1 expression in T cells (16, 17). Therefore, we sought to explore the mechanism of IL-2-dependent PD-1 regulation. The reduction of PD-1 by IL-2 was dose-dependent, and 1 ng ml$^{-1}$ IL-2 achieved the optimum reduction (Fig. 1C). IL-2 enhanced T-cell activation, and the proportion of the CD25$^+$ population became massive after 28 h compared to the T-cell culture without IL-2 (Fig. 1D). This proportional change was not the reason of PD-1 reduction in the IL-2-treated T-cell culture since a comparison within the CD8$^+$ CD25$^+$ population also demonstrated a notable PD-1 reduction by IL-2 treatment (Fig. 1E). In addition, these activated T cells should have received TCR stimulation plus IL-2 signaling because T-cell culture with IL-2 alone was yet to start vigorous cell proliferation (Supplementary Figure 2). Such IL-2-dependent PD-1 regulation was functional in the stimulation of not only CD8$^+$ but also CD4$^+$ T cells (Fig. 1F). This inhibitory effect of IL-2 was not observed for LAG3, another activation-induced immune inhibitory molecule (Fig. 1G), suggesting an underlying mechanism which can specifically destroy PD-1 induction.

Although IL-2 reduced PD-1 levels, these T cells still remained PD-1-positive. We examined whether this extent of PD-1 modulation was of any significance in terms of T-cell sensitivity to this immunosuppressive mechanism. In the comparison of PD-1$^{+/+}$ T cells and PD-1$^{+/-}$ cells, the reduced PD-1 expression due to the haploinsufficiency of the PD-1 allele significantly affected T-cell sensitivity to PD-L1. Namely, T cells from PD-1$^{+/-}$ mice expressed intermediate levels of PD-1 upon activation compared to PD-1$^{+/+}$ T cells (Fig. 2A). These T cells showed different degrees of sensitivity to PD-L1-expressing cells. The comparison of IFN-γ production in the presence and absence of anti-PD-1 blocking antibody indicated that PD-1 stimulation of PD-1$^{+/+}$ T cells reduced the cytokine production to 40%, while PD-1$^{+/-}$ T cells retained 80% of the activity (Fig. 2B). This result suggested that the IL-2-dependent PD-1 reduction from the normal level may also affect the sensitivity to PD-L1-expressing cells.

To examine the impact of IL-2-dependent PD-1 reduction in T-cell activities, we tested their cytotoxicity to PD-L1-expressing target cells. The cytotoxicity of control CD8$^+$ T cells was strongly impaired by PD-1 signaling as indicated by the striking rescue of cytotoxicity by anti-PD-1 mAb; however, IL-2-treated CD8$^+$ T cells remained highly cytotoxic regardless of PD-L1 expression by target cells (Fig. 2C and D). The cytokine-producing activity in the co-culture with PD-L1-expressing cells reproduced the same trend. When re-stimulated immediately after the primary T-cell stimulation, control CD8$^+$ T cells were more sensitive to immunosuppression by PD-L1-expressing cells (40% reduction in IFN-γ production) than IL-2-treated T cells (15% reduction), although the amount of IFN-γ from IL-2-treated CD8$^+$ T cells was 100 times greater than control T cells (data not shown). We further examined PD-1-mediated inhibition of cytokine-producing activity in the extended T-cell culture. After 28-h primary

activation, CD8$^+$ T cells were re-stimulated for 12 h in the presence or absence of IL-2. After this re-culture, PD-1 levels in T cells with IL-2 were lower than in those without IL-2 (Fig. 2E and F). T cells without IL-2 were prone to the immunosuppression in the interaction with PD-L1-expressing cells as indicated by the significant increase of IFN-γ production by PD-1 blockade. However, in the same setting, PD-1 blockade did not significantly enhance IFN-γ production from IL-2-treated T cells, suggesting that PD-1 reduction by IL-2 resulted in T-cell resistance to this immunosuppressive mechanism (Fig. 2G). Although IL-2-treated T cells still produced a larger amount of IFN-γ than those without IL-2, the difference was diminished to several-fold in this setting. Intracellular staining of IFN-γ confirmed the relative resistance of IL-2-treated T cells to PD-1 stimulation (Fig. 2H and I). These results indicate that IL-2 can reduce PD-1 expression in activated T cells and consequently increases resistance of T cells to PD-1-mediated immunoregulation.

### Transcription factors NFAT1 and STAT5A regulate PD-1 gene expression

We next asked the underlying mechanism of the IL-2-dependent reduction of PD-1 levels. PD-1 induction in activated T cells does not persist for long and is eventually down-regulated after the disappearance of TCR stimulation. Recently, FBXO38 was found to be responsible for the turnover of PD-1 protein by ubiquitination and subsequent proteasomal degradation (18). In the same study, IL-2 was found to induce FBXO38 and promote PD-1 degradation. FBXO38 mRNA was reported to start increasing after 2 days with IL-2; however, in our experiment, IL-2 clearly reduced PD-1 levels in 1 day without induction of FBXO38 mRNA (Supplementary Figure 3). Moreover, accelerated degradation of PD-1 protein was unlikely to be the principal mechanism of IL-2-dependent PD-1 reduction in our setting because proteasome/lysosome inhibitors failed to significantly reverse PD-1 reduction in IL-2-treated T cells (Supplementary Figure 3). As an alternative explanation, the analysis of PD-1 mRNA levels showed that IL-2 could inhibit PD-1 gene expression (Fig. 3A), suggesting a negative regulatory mechanism at the transcriptional level.

NFAT1 and NFAT2 have been shown to mediate transcriptional up-regulation of PD-1 during T-cell activation (10, 19). We sought to investigate if IL-2 signaling affected NFAT-dependent PD-1 induction. For this purpose, we co-transfected a PD-1 promoter-luciferase reporter vector together with constitutively active NFAT1 and/or STAT5A into HEK293 cells. Co-transfection of constitutively active NFAT1 induced the promoter activity (Fig. 3B). To mimic the IL-2 receptor signaling, constitutively active STAT5A was co-transfected to the cells. Although STAT5A acted as an activator in PD-1 gene regulation, its transcriptional potential was much weaker than that of NFAT1 (Fig. 3B). Importantly, co-transfection of both NFAT1 and STAT5A reduced luciferase activities compared to NFAT1 alone (Fig. 3B).

### Competition of transcription factors regulates PD-1 transcription

This result led us to hypothesize the existence of an NFAT1 and STAT5A competition site in the PD-1 promoter region given that these two transcriptional factors share overlapping consensus

**A**



**B**



**C**



**D**



**E**



**F**



**G**



**H**



**I**
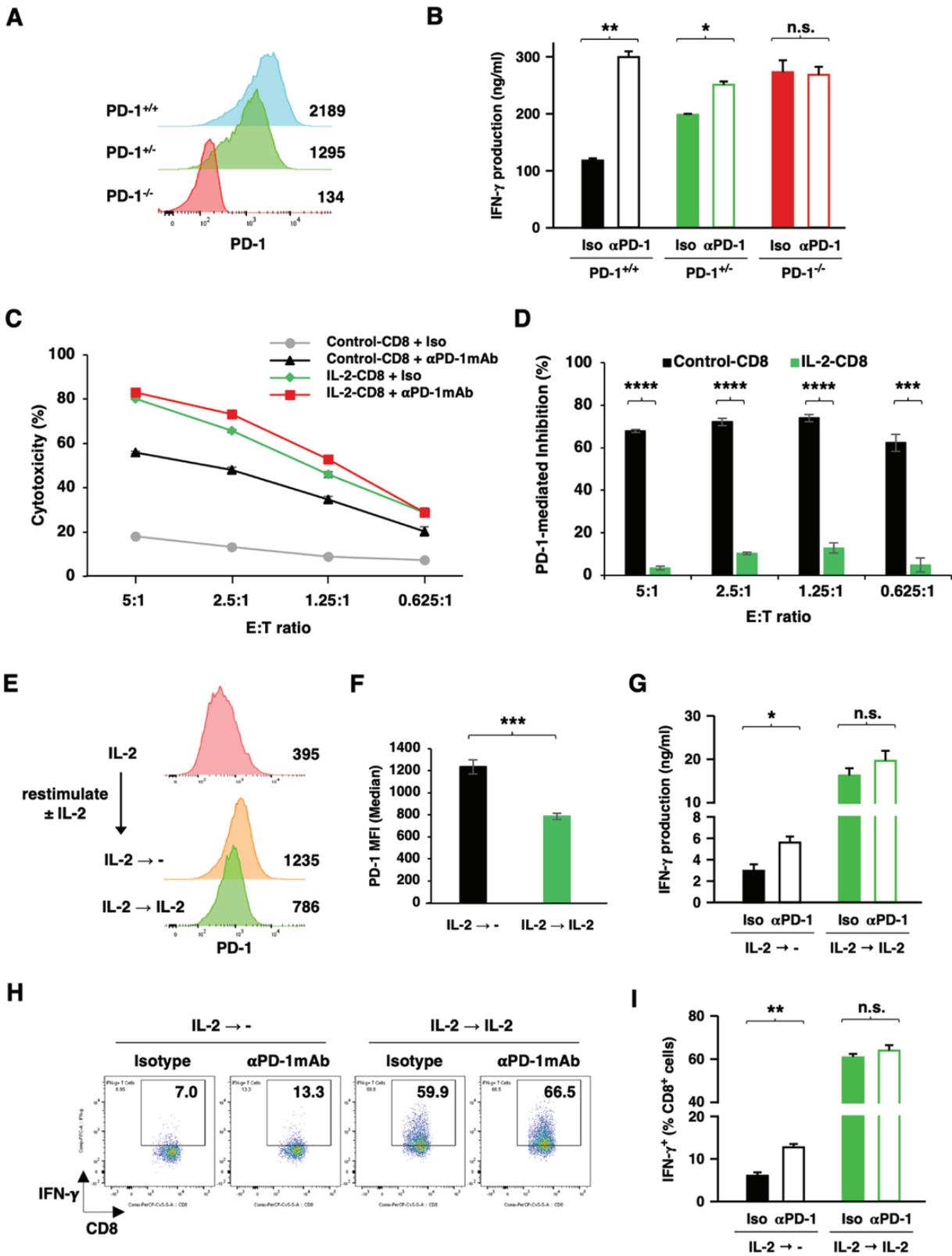


**Fig. 2.** Reduction of PD-1 expression by IL-2 leads to insensitivity to PD-L1-induced immunosuppression. (A) PD-1 expression after 24-h stimulation of CD8$^+$ T cells from wild-type (PD-1$^{+/+}$), PD-1 heterozygous (PD-1$^{+/-}$) and knockout (PD-1$^{-/-}$) mice. Numbers in the panels represent the mean fluorescence intensity of the PD-1 signal. (B) Degree of PD-1-mediated immunosuppression depends on the surface PD-1 levels. Activated CD8$^+$ T cells in the panel A were re-stimulated using PD-L1-expressing IIA1.6 cells in the presence of anti-PD-1 blocking mAb or
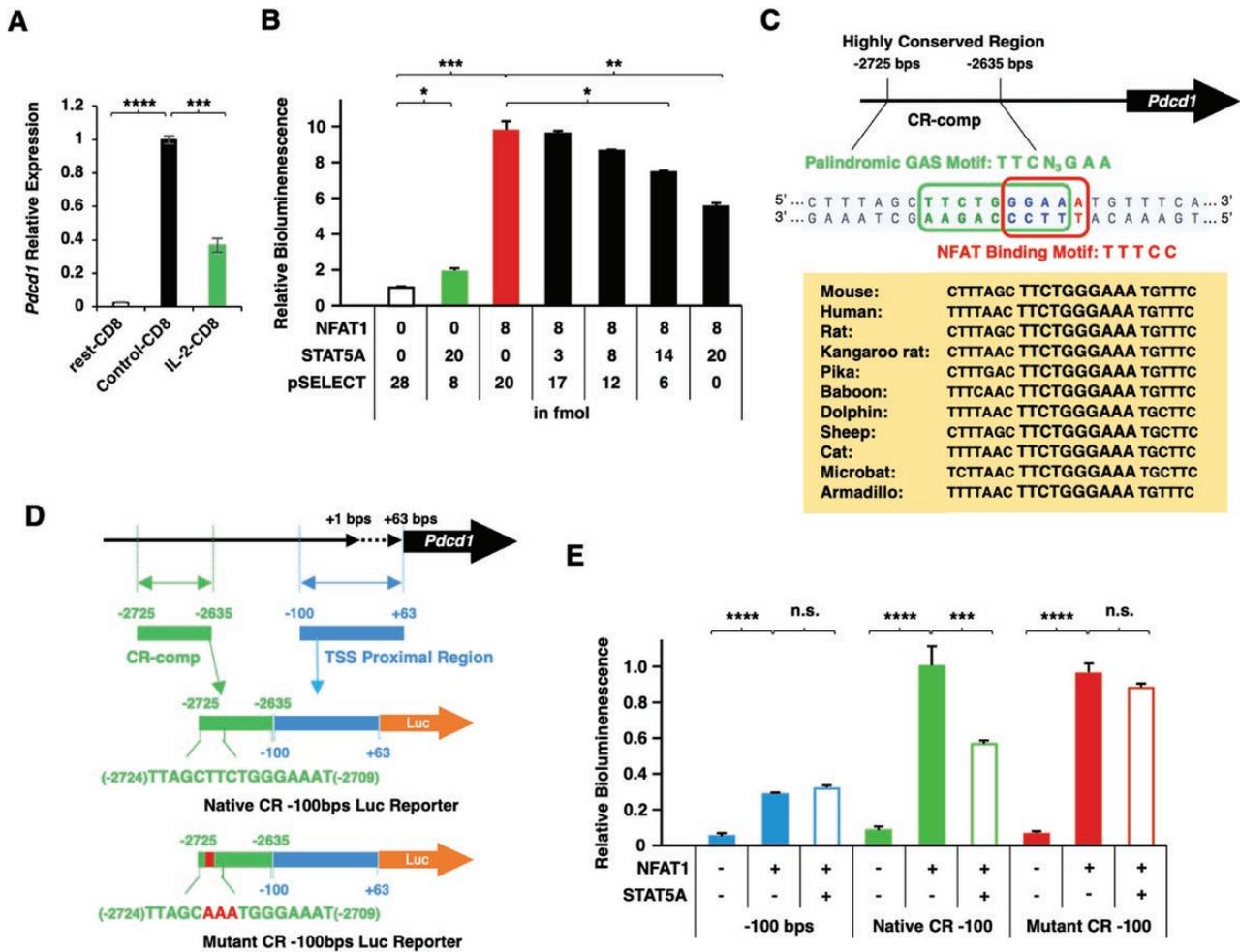
**Fig. 3.** IL-2 inhibits PD-1 transcription. (A) *Pdcd1* mRNA levels in activated CD8+ T cells after 28 h. (B) Luciferase reporter assay of PD-1 promoter (−3000 to +63 bp). Co-transfection of constitutively active NFAT1-induced transcription, but constitutively active STAT5A blocked the NFAT1-dependent up-regulation. (C) The highly conserved region between −2725 and −2635 bp in the PD-1 promoter. This region (CR-comp) contains an overlap of the STAT binding motif (GAS motif) and the NFAT binding motif. (D, E) The CR-comp was combined with the −100 to +63 bp PD-1 transcription start site in the luciferase reporter construct. Constitutively active NFAT1-induced transcription with native CR-comp, and STAT5A inhibited the up-regulation. The inhibitory effect of STAT5A was sequence-specific since it did not interfere with NFAT1-induced transcription with mutant CR-comp in which the STAT binding motif was disrupted. Data represent the means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, two-tailed student's *t*-test analysis. Data shown here are representative of three independent experiments.

binding motifs (Fig. 3C). In the analysis of the PD-1 promoter sequence, a highly conserved region between −2725 and −2635 bp was found to contain a STAT binding motif overlapped with the NFAT binding site. In order to investigate a possible competition over this site, the −2725 to −2635 bp region, named the conserved region competition (CR-comp), was combined with the −100 to +63 bp of the PD-1 transcription start site in the luciferase reporter construct (Fig. 3D). Constitutively active

NFAT1 strongly induced luciferase activity, and co-transfection of STAT5A suppressed the NFAT1-induced transcription, suggesting that STAT5A can compete with NFAT1 in this region (Fig. 3E). Site-specific mutagenesis was introduced to disrupt the STAT5A binding motif (TTCxxxGAA → AAAxxxGAA) (Fig. 3D), and while NFAT1 could induce transcriptional activation, STAT5A lost the capacity to outcompete the NFAT1 promoter activity (Fig. 3E). This result suggests that STAT5A competes

isotype control antibody. (C–I) The reduction of PD-1 expression by IL-2 treatment led to insensitivity to PD-L1-expressing cells. Cytotoxicity of CD8+ T cells after activation in the presence or absence of IL-2 against PD-L1-expressing IIA.1.6 cells. PD-1-dependent inhibition (%) = (cytotoxicity with isotype control)/(cytotoxicity with anti-PD-1 blocking mAb) × 100 (C, D). Cytokine-producing activity of CD8+ T cells when stimulated using PD-L1-expressing cells (E–I). Initially, CD8+ T cells were stimulated with anti-CD3/CD28 mAb-conjugated microbeads for 28 h in the presence of IL-2. Activated T cells were re-stimulated with the same beads in the presence or absence of IL-2 for 12 h, and this brief switch sufficiently created a difference in PD-1 levels (E, F). Subsequently, IFN-γ-producing activities of T cells were evaluated in a co-culture with PD-L1-expressing IIA1.6 cells in the presence of anti-PD-1 blocking mAb or isotype control. IFN-γ levels in the supernatant were determined after 6 h (G). The proportions of IFN-γ-producing T cells were also determined by intracellular staining (H, I). Data represent the means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, two-tailed student's *t*-test analysis. Data shown here are representative of three independent experiments.
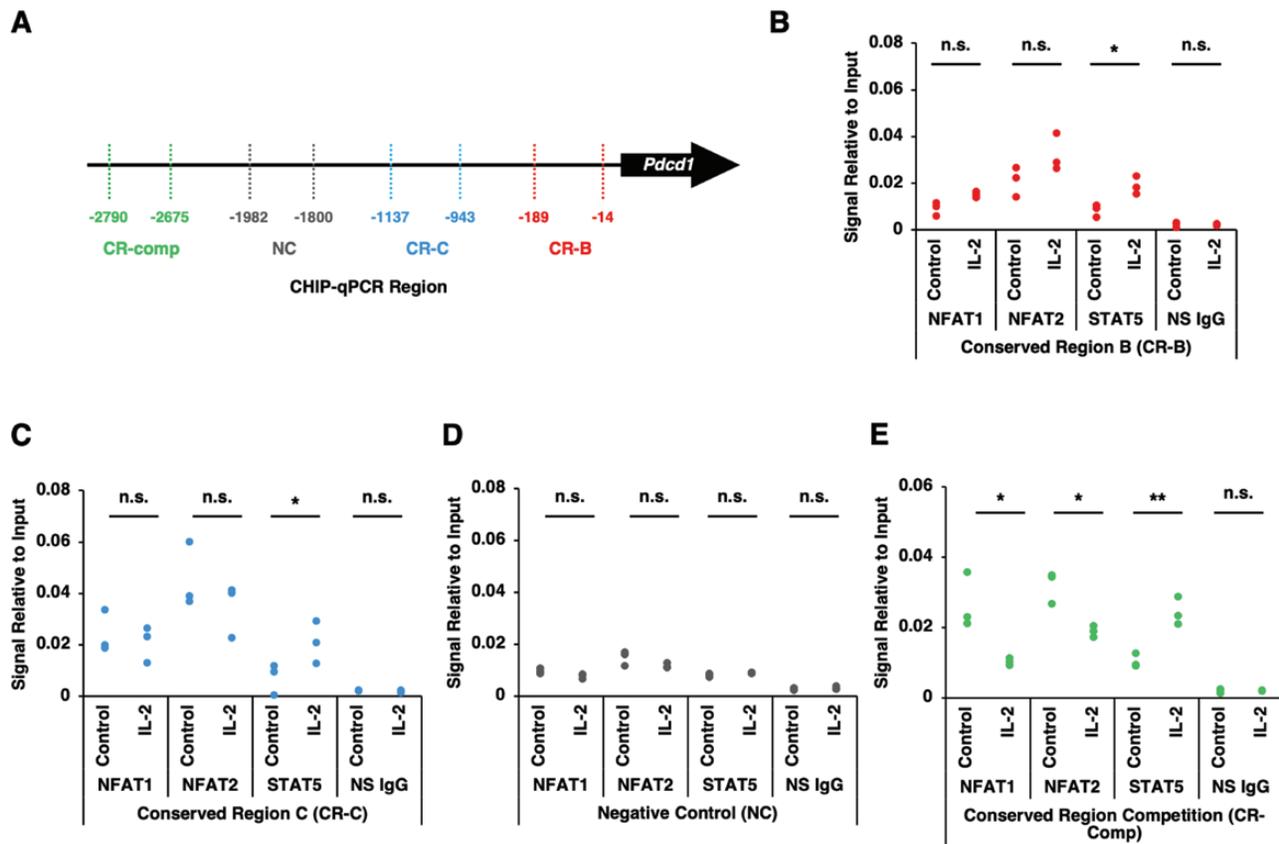
**Fig. 4.** STAT5 outcompetes NFAT for binding to CR-comp in IL-2-treated CD8⁺ T cells. (A) ChIP–qPCR analysis of NFAT binding regions: conserved region B (CR-B), conserved region C (CR-C) and CR-comp. NC means negative control. (B–E) Mouse CD8⁺ T cells were stimulated in the presence or absence of IL-2 for 28 h and were subjected to ChIP–qPCR for the binding of NFAT1, NFAT2 and STAT5. *$P < 0.05$, ** $P < 0.01$, two-tailed student's *t*-test analysis. Data shown here are representative of three independent experiments.

with NFAT1 for the −2724 to −2709 bp overlapped binding site in a sequence-specific manner.

To further examine the implication of the STAT5-NFAT competition in the PD-1 regulation in T cells, we performed the ChIP coupled with quantitative PCR (ChIP–qPCR). In this assay, we analyzed how IL-2 exposure of CD8⁺ T cells changed NFAT/STAT5 binding to the PD-1 promoter. For the NFAT-dependent transcriptional activation of PD-1, previous studies have identified two NFAT binding regions, CR-B and conserved region C (CR-C) (10, 20). In order to gain a comparable understanding of these regions, the assay was conducted with CR-B, CR-C and CR-comp plus a negative control (Fig. 4A). The result highlighted the significance of NFAT1 and NFAT2 binding in all three regions (Figure 4B–E). While IL-2 treatment of T cells did not affect NFAT binding to CR-B and CR-C, it did significantly decrease NFAT interaction with CR-comp concomitant with the increase of STAT5 binding. Taken together, the current results suggest that IL-2 inhibited PD-1 induction in T cells by the competition between STAT5 and NFAT over the overlapped binding sequence in the CR-comp region.

## Discussion

While PD-1 is normally undetectable on most of resting T cells, it is up-regulated upon T-cell activation, serving as a negative feedback mechanism of T-cell activities. TCR

signaling has been shown to induce PD-1 transcription via NFAT. The PD-1 promoter contains multiple NFAT binding sites that can positively regulate PD-1 transcription (10, 11, 21). In contrast, T-bet and Blimp-1 serve as negative regulators of PD-1. Chronic stimulation of T cells in viral infection reduces T-bet expression and subsequently increases PD-1 levels in exhausted T cells (22, 23). Blimp-1 deficiency increases PD-1 expression in chronic T-cell stimulation since Blimp-1 can repress NFAT-mediated PD-1 induction through chromatin remodeling (24).

Cytokines can also affect PD-1 levels on T cells. In the current study, when added along with T-cell stimulation, cytokines modified PD-1 levels in different directions (Fig. 1). The augmentation of PD-1 expression by IL-12 and TGF-β is consistent with previous reports that showed transcriptional up-regulation of PD-1 by these cytokines (13, 14, 25). Conversely, IL-2 and IL-4 diminished PD-1 levels in activated T cells. IL-2 has been reported to down-regulate PD-1 expression *in vitro* (18, 26), and IL-2-treated mice had CD8⁺ T cells with lower levels of PD-1, which were resistant to PD-L1-expressing target cells (16, 17). Correspondingly, IL-2-treated CD8⁺ T cells in our experiment also showed reduced sensitivity to immunosuppression by PD-L1-expressing target cells (Fig. 2). Such a correlation between PD-1 levels and sensitivity to PD-1 ligands was also shown in PD-1 up-regulation by glucocorticoid (27). The haploinsufficiency of the PD-1 allele provided more evidence for the direct correlation of PD-1

expression levels with the sensitivity of activated T cells to PD-1-dependent immunoregulation. PD-1$^{+/-}$ T cells expressed lower levels of PD-1 upon activation than PD-1$^{+/+}$ T cells, and the difference in PD-1 levels was reflected into the extent of T-cell suppression with PD-L1-expressing targets, which effectively suppressed activities of PD-1$^{+/+}$ T cells but much less in PD-1$^{+/-}$ T cells (Fig. 2). The expression levels of PD-1 have a significant impact on physiological immunoregulation since PD-1$^{+/-}$ NOD mice spontaneously develop type 1 diabetes faster and more intensively than PD-1$^{+/+}$ NOD mice (6).

In the current study, IL-2 signaling triggered a negative regulation that could counteract the TCR signaling-mediated PD-1 induction. We found the interference of PD-1 transcription by activated STAT5 in the IL-2 receptor signaling pathway. Both NFAT1 and STAT5 can be independent positive regulators of PD-1 gene expression; however, their transcriptional potencies were substantially different (Fig. 3). Consistent with this result, NFAT deficiency critically impaired PD-1 up-regulation in T-cell activation (19), while STAT5 deletion did not decrease PD-1 levels (28). STAT5 was previously suggested to inhibit NFAT1-dependent gene transcription in breast cancer cells, but the mechanism of action was unclear (29). Our analysis of the PD-1 promoter identified the conserved region CR-comp in which NFAT1 and STAT5 have a unique overlapping binding site. This region represents one of the DNase I-hypersensitivity sites in activated, but not naive, T cells (30). Since transcriptional activation by STAT5 was much weaker than NFAT1 (Fig. 3), the competitive NFAT1 replacement by STAT5 would hamper full activation of PD-1 gene by TCR signaling. For the NFAT1-dependent PD-1 regulation, the deletion of the cis-regulatory element CR-C critically impaired PD-1 expression in T cells *in vitro* and *in vivo* (11). Our ChIP–qPCR assay indicated that both NFAT1 and STAT5 interacted with CR-comp as well as CR-C (Fig. 4), consistent with a previous report (19). Interestingly, the analysis of IL-2-treated T cells pointed out that activated STAT5 outcompeted NFAT1 for binding at CR-comp, but not CR-C, and thus diminished PD-1 up-regulation by TCR signaling. This result suggests that CR-comp may also play a major role in the NFAT1-dependent PD-1 up-regulation along with CR-C. LAG3 is another T-cell activation-induced immune inhibitory molecule that is under the control of NFAT (19). IL-2 negatively regulated PD-1 without any effect on LAG3 levels (Fig. 1). The promoter analysis of LAG3 gene shows the existence of NFAT and STAT transcription binding sites in the open region but, unlike PD-1, with no overlap.

Another cytokine that could diminish PD-1 expression in stimulated T cells was IL-4 (Fig. 1). In addition, IL-7 has been shown to reduce PD-1 in stimulated T cells *in vitro* and *in vivo* (31–33). Receptors for IL-2, IL-4 and IL-7 share the same common gamma subunit. JAK3 binds to the common gamma chain and subsequently activates STAT5 (34, 35). This signaling mechanism might have participated in the PD-1 regulation in the presence of these cytokines.

In conclusion, the eviction of activated NFAT1 by STAT5 under the cytokine signaling pathway was raised, leading to the proposal of a possible involvement of transcription factor competition in the regulation of PD-1 expression. A decrease in PD-1 levels at the physiological range of expression had a significant impact on the sensitivity of immune cells to PD-1 ligand-expressing targets. The transduction of constitutively active STAT5 was shown to enhance the anti-tumor performance of T cells (36, 37). Those T cells with active STAT5 expressed high levels of IFN-γ and granzyme B in the tumor microenvironment, and interestingly, their PD-1 levels were slightly lower than T cells without the constitutively active STAT5 (36). Besides therapeutic treatment to enhance the IL-2 signaling pathway, it is also possible that IL-2 from activated immune cells autonomously affects PD-1 levels. If endogenous IL-2 levels in the local microenvironment reach high enough levels, this mechanism of PD-1 regulation may have some significance in physiological immune regulation. Further studies on the molecular mechanisms of PD-1 regulation may promote our understanding of the endogenous control of T-cell activities in cancer and inflammatory disorders.

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